Vol. 98, No. 1, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1981 Pages 176-183

NEOCARZINOSTATIN: CONTROLLED RELEASE OF CHROMOPHORE AND ITS INTERACTION WITH DNA

Gundram Jung and Wolfgang Köhnlein

Institut für Strahlenbiologie der Westfälischen Wilhelms Universität, 4400 Münster, Germany

Received November 6,1980

SUMMARY

Reinterpreting a previously established model of neocarzinostatin-DNA interaction in view of new developments, we present evidence that release of non protein active chromophore is the first step in neocarzinostatin action. Once released the chromophore may react with DNA to form strand breaks or/and undergo inactivation. This leads to a constant disturbance of the equilibrium between native and dissociated protein resulting in a complete overall dissociation and inactivation of the drug. The time course of this inactivation determines the biological availability of the active chromophore and has been shown to be rather fast under conditions occurring in vivo. It can be influenced by adding chromophore free protein (apoprotein), by changing the stability of released chromophore and also by altering the conformation of the apoprotein.

INTRODUCTION

Neocarzinostatin is a single chain acidic protein of molecular weight 10,700 and known amino acid sequence (1). NCS exhibits antitumor activity in a variety of experimental (2) and clinical tumors (3,4). It causes degradation of DNA in vivo (5) and if sulfhydryls are present in vitro (6). There is a large body of additional information strengthening the notion that DNA is a main target of the drug (7). In 1974 Kikuchi et al. isolated a protein very similar to NCS concerning amino acid composition with a slightly lower pI point, which lacks the biological activity of native protein. The inactive form was thought to be a precursor of NCS and was referred to as pre-NCS. It antagonizes the in vivo effects (2) as well as the DNA strand breaking activity of the drug in vitro and has been obtained from NCS by a variety of different treatments (8).

Abbreviations: NCS, neocarzinostatin; SSC, standard saline citrate; CD, circular dichroism; Tris, Tris(hydroxymethyl)-aminomethan.

0006-291X/81/010176-08$01.00/0

Previously we have proposed a model in which NCS-DNA interaction proceeds via a short lived active form of the drug which either reacts with DNA to form strand breaks or decays into an inactive state thought to be pre-NCS (9). All attempts, however, to detect direct binding of the protein to DNA failed (10; unpublished results). At the same time it became obvious that the active NCS contains a non protein chromophore which is very unstable in aqueous solution and can be isolated by methanol extraction (11). The isolated chromophore is biologically active (12,13), binds to DNA and induces strand breaks in the presence of mercaptoethanol (12,14). The chromophore free protein has the same pI point as pre-NCS and can associate with active chromophore to form native NCS. It was therefore concluded that pre-NCS is the apoprotein of a native NCS chromophore complex, preventing rapid inactivation of active chromophore in aqueous solution (12).

In view of these new developments we suppose that the released active chromophore is the short lived active form proposed in our model. Indeed, the kinetic data presented here are explained best by the assumption that release of chromophore is the first step in NCS DNA interaction followed either by reaction of active chromophore with DNA to form strand breaks or/and its rapid inactivation.

MATERIALS AND METHODS

Neocarzinostatin (lot Nr. 770206) clinical form in 0.015 M sodium acetate buffer, pH 5 originated from Kayaku Antibiotics Research Co. and was kindly supplied to us by Dr. W.T. Bradner of Bristol Laboratories.

T2 DNA preparation, sucrose gradient sedimentation, isoelectric focusing and optical measurements have been reported in detail elsewhere (8,9).

Treatment of T2 DNA with NCS: Reaction mixtures of a total volume of 170 µl contained 50 µl DNA (140 µg/ml) in SSC, 0.025 M Tris pH 7.5, and the various drug , mercaptoethanol , urea, and isopropanol concentrations as indicated in the legends of the figures. For inactivation kinetics reaction mixtures were preincubated containing a corresponding volume of SSC instead of DNA. After indicated times aliquots were given to DNA and proper amounts of mercaptoehtanol were added to assure complete reaction of the remaining drug within one hour. Samples not preincubated were taken as reference. All handling was in the dark.

Preparation of chromophore free NCS was by heat inactivation of native drug for 20 hours at 54°C. The dissociated protein was dialysed against 0.025 M Tris pH 7.5. CD spectra of native inactivated, and dialysed protein were monitored (data not shown). The CD spectrum of heat inactivated dialysed protein is completely identical with the known spectrum of chromatographically isolated apoprotein (15).

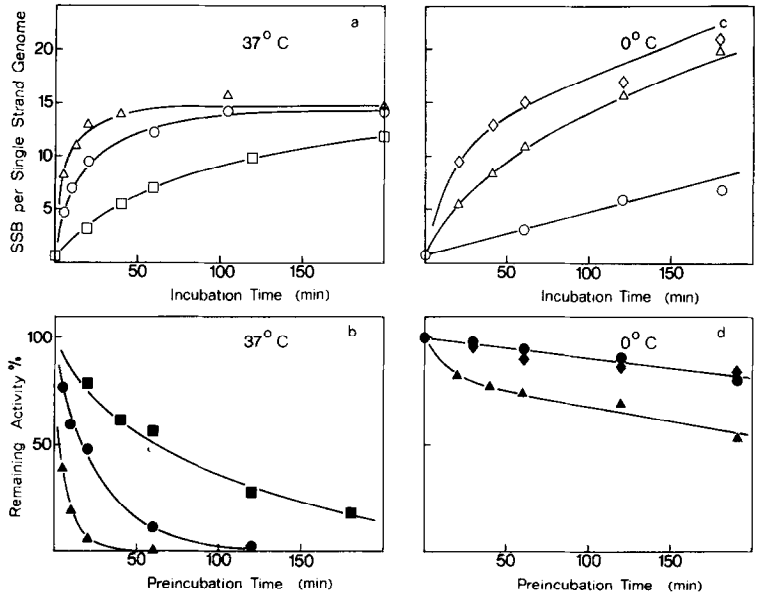

Fig. 1. Time course of NCS activation (a,c; open symbols) and inactivation (b,d; closed symbols). Reaction and preincubation mixtures contain 1.2 µg/ml NCS; 0.6 mM mercaptoethanol (○,●) and 3 M urea (△,▲) or apoprotein 3.6 µg/ml (□,■) or 1.2 M isopropanol (◇,◆). Mean values of at least two independent measurements.

RESULTS

In Fig. 1 the time course of inactivation of the native protein is shown for various conditions. The remaining activity at any given time point is strongly correlated to the amount of undissociated NCS found on isoelectric focusing gels (Fig. 2, Fig. 3). This inactivation and conversion to the lower pI point is interpreted to be due to fast further reactions of the released chromophore, therefore, driving the equilibrium between native and dissociated protein to complete dissociation. Consequently upon addition of apoprotein one would expect a decrease of the inactivation rate due to alteration of the equilibrium. This is indeed observed as can be seen in Fig. 1b. Moreover the time course of drug action under the same conditions shows a corresponding decreased initial strand break rate (Fig. 1a). However, the final extent of DNA degradation was not changed (Tab. 1). In Fig. 4 the remaining activity is plotted versus preincubation time for various initial drug con-

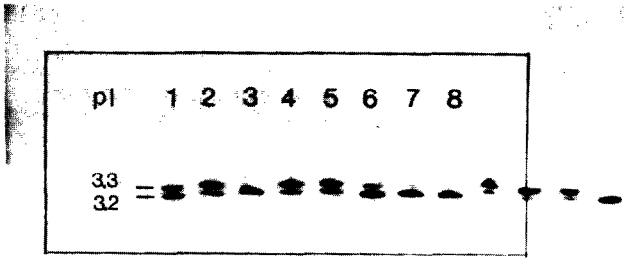

Fig. 2. Isoelectric focusing of NCS (190 µg/ml in 0.05 M Tris, pH 7.5) after various treatments for 3 h: 1. 7 M urea, 37°C; 2. no urea, 37°C; 3. 3 M isopropanol, 37°C; 4. no isopropanol, 37°C; 5. 4.5 mM mercaptoethanol, 0°C; 6. same but 20°C; 7. same but 37°C; 8. same but 54°C.

centrations. Such an unusual concentration dependence clearly indicates that the involved reaction is not of first order.

Some agents can influence the overall dissociation rate either by changing chromophore release or altering its further reactions. In the latter case one would expect in addition to different initial strand break rates also a variation in the final extent of DNA-degradation. Since after chromophore release various reactions are possible, the plateau level of DNA degradation yields information on the fraction of chromophore molecules producing strand breaks in contrast to those which are just inactivated.

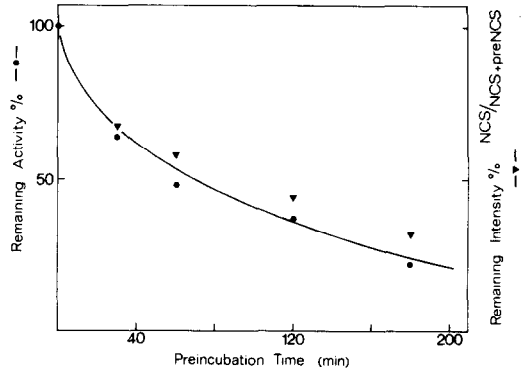

Fig. 3. Remaining activity of NCS (190 µg/ml) preincubated with 4.5 mM mercaptoethanol at 37°C (●) and remaining intensity of the active NCS band obtained by microdensitometric tracings of isoelectric focusing gel (gel not shown) (▼). Corresponding aliquots taken from samples treated identically.

Table 1.

Number of single strand breaks at the plateau level are given after incubations for 14 hours. Reaction conditions as in Fig. 1; given values are mean values of at least three independent measurements.

Temperature	Added materials			
	None	Urea 3 M	Isoprop. 1.2 M	Apo NCS 3.6 µg/ml
0° C	-	26.6	35.6	-
20° C	18.3	18.7	28.0	-
37° C	14.0	14.2	20.6	14.2

As we have shown previously (9) increasing sulfhydryl concentrations increase initial strand break rate and final plateau level. As illustrated in Fig. 1c and Tab. 1 lowering the temperature decreases the initial slope, however, a remarkable increased plateau level is obtained. In the presence of denaturing agent urea only the initial slope is increased, indicating that changing the conformation of apoprotein influences release of chromophore. This is especially well pronounced at 0°C (Fig. 1c), where NCS alone has only a reduced activity while the isolated chromophore remains fully active (12). In the presence of isopropanol the final extent of DNA degradation is enhanced as well (Tab. 1), showing that this agent also influences the further reactions of the chromophore. As far as

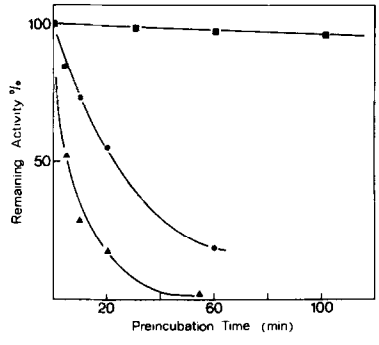

Fig. 4. Time course of inactivation of NCS at 37°C in the absence of sulfhydryls for various initial drug concentrations. Samples diluted in 0.05 M Tris, pH 7.5 (■ 13o µg/ml; ● 1.2 µg/ml; ▲ 0.3 µg/ml).

the effects of temperature and isopropanol are concerned, our results are in good agreement with the findings of Povirk et al. (14) that low temperature and high isopropanol concentration stabilize the isolated chromophore.

DISCUSSION

The results presented here lead to the assumption that release of chromophore is the first step in drug action. The chromophore once released may undergo further reactions therefore leading to complete dissociation of native NCS, despite the reported high affinity (14) of the active chromophore to apoprotein. Povirk et al. have also observed that inactivation of the isolated chromophore may proceed via its spontaneous degradation in aqueous solution forming an inactive species with marked fluorescence at 490 nm and rather low affinity to apoprotein. In addition in the presence of mercaptoethanol a specific chromophore thiol interaction may take place generating a chromophore which is not able to form strand breaks but seems to bind to DNA. A specific drug thiol interaction is indicated from our observations comparing CD activity of NCS inactivated by various methods (data not shown). However, the exact reaction sequence in which the released active chromophore reacts with sulhydryls and DNA remains to be clarified.

At 37°C our results show a complementary time course of strand break production and remaining activity (Fig. 1a,1b). At 0°C, however, added isopropanol activates the drug while no corresponding inactivation was observed (Fig. 1c, 1d). These results may indicate that in this case DNA accelerates chromophore reaction. The fast inactivation at 37°C and pH 7.5 especially at low drug concentrations even in the absence of thiols may indicate a rather short time interval of NCS action in vivo. In general it might be of some interest for the clinical application of the drug that the biological availability of active chromophore can be manipulated by the changing of its stabilizing "cage" (apoprotein) or adding surplus apoprotein.

The effects of various agents on the activity of the native protein including the previously reported stabilizing effect of

low pH (9) are in agreement with the known effects of those agents on the isolated chromophore (14). Taken together this provides additional evidence for the role of released chromophore during action of native protein.

Nevertheless it should be noted that temperature effect on strand break plateau level is contrary to the one reported here, if high drug concentrations in the absence of sulfhydryls are used (9). Taken the fact that sulfhydryls are essential for activation of the isolated chromophore and that isopropanol has no effect on NCS action in the absence of sulfhydryls (16), the reversed temperature effect might indicate a different reaction mechanism under those conditions, possibly without involvement of the released chromophore.

ACKNOWLEDGEMENTS

This work was supported in part by a grant from the Deutsche Forschungsgemeinschaft (Kö 347/7-9). The authors are indebted to Dr. Lezius for providing access to the CD spectrograph and to Dr. W.T. Bradner for a gift of clinical NCS.

REFERENCES

1. Meienhofer, J., Maeda,H., Glaser, Ch.B., Czombos, J., and Kuromizu, K. (1972) Science 178,875-876.
2. Kikuchi, M., Shoij, M., Ishida, N. (1974) J. Antibiot. 27, 766-774.
3. Legha, S.S., van Hoff, D.D., Rosencweig, M., Abraham, D., Slavik, M., and Muggia, M. (1976) Oncology 33, 265-270.
4. Falkson, G., Von Hoff, D., Kaassen, D., Du Plessis, H., Van Der Merwe, C.F., Van Der Merwe, A.M., and Carbone, P.P. (1980) Cancer Chemoth. Pharmacol. 4, 33-36.
5. Tatsumi, K., Nakamura, T. and Wakisaka, G. (1974) Gann 65, 459-461.
6. Beerman, T.A., and Goldberg, I.H. (1974) Biochem.Biophys. Res.Commun. 59, 1254-1261.
7. Beerman, T.A., Poon, R. and Goldberg, I.H. (1977) Biochim. Biophys. Acta, 475, 294-306.
8. Jung, G., Lewis, R.S. and Köhnlein, W. (1980) Biochim. Biophys. Acta 608, 147-153.
9. Lewis, R.S., Jung, G., and Köhnlein, W. (1980) Biochim, Biophys. Acta 608, 138-146.
10. Maeda, H., Aikawa, S. and Yamashita, A. (1975) Cancer Research 35, 554-559.
11. Napier, M.A., Holmquist, B., Strydom, D.J., and Goldberg, I.H. (1979) Biochem.Biophys.Res.Commun. 89, 635-642.

12. Kappen, L.S., Napier, M.A. and Goldberg, I.H. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 1970-1974.
13. Suzuki, H., Miura, K., Kumada, Y., Takeuchi, T., and Tanaka, N. (1980) Biochem.Biophys.Res.Commun. 94, 255-261.
14. Povirk, L. and Goldberg, I.H. (1980) Biochemistry 19, (in press).
15. Napier, M.A., Kappen, L.S., and Goldberg, I.H. (198) Biochemistry 19, 1767-1773.
16. Kappen, L.S., and Goldberg, I.H. (1979) Biochemistry 18, 5647-5653.